Plant Physiol. (1996) 112: 811–819

Genetic Engineering of Plant Secondary Metabolism[1]

Accumulation of 4-Hydroxybenzoate Glucosides as a Result of the Expression of the Bacterial *ubiC* Gene in Tobacco

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The *ubiC* gene of *Escherichia coli* encodes chorismate pyruvate-lyase, an enzyme that converts chorismate into 4-hydroxybenzoate (4HB) and is not normally present in plants. The *ubiC* gene was expressed in *Nicotiana tabacum* L. plants under control of a constitutive plant promoter. The gene product was targeted into the plastid by fusing it to the sequence for the chloroplast transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Transgenic plants showed high chorismate pyruvate-lyase activity and accumulated 4HB as β-glucosides, with the glucose attached to either the hydroxy or the carboxyl function of 4HB. The total content of 4HB glucosides was approximately 0.52% of dry weight, which exceeded the content of untransformed plants by at least a factor of 1000. Feeding experiments with [1,7-$^{13}C_2$]shikimic acid unequivocally proved that the 4HB that was formed in the transgenic plants was not derived from the conventional phenylpropanoid pathway but from the newly introduced chorismate pyruvate-lyase reaction.

Genetic engineering has allowed the production of plants with an altered content of secondary metabolites. Because secondary metabolites are important in the defense of plants against pathogens, such engineered plants may show an increase in resistance against pathogens. For example, the expression of a stilbene synthase from *Vitis vinifera* in tobacco (*Nicotiana tabacum*) led to the accumulation of stilbenes and thereby to an increased resistance to *Botrytis cinerea*, providing direct evidence of the role of stilbenes as phytoalexins (Hain et al., 1993). In other experiments the accumulation of secondary metabolites, which are already produced in the untransformed plants, has been increased by the overexpression of structural genes encoding biosynthetic enzymes. Expression of hyoscyamine 6-hydroxylase in *Atropa belladonna* plants, for example, led to a strong increase of scopolamine production in the transgenic plants (Yun et al., 1992), and expression of a bacterial Lys decarboxylase in tobacco increased the production of the diamine cadaverine (Fecker et al., 1993). Such experiments may prove useful for the production of secondary metabolites of pharmaceutical importance, both by intact plants and by plant cell cultures.

The commercial production of a pharmaceutical substance by plant cell culture has already been realized on an industrial scale in the case of shikonin, a naphthoquinone pigment with antibacterial, antiphlogistic, and wound-healing properties that is obtained from cell cultures of *Lithospermum erythrorhizon* (Tabata and Fujita, 1985). Shikonin is biosynthetically derived from 4HB and geranylpyrophosphate (Heide and Tabata, 1987). Using feeding experiments with [1,7-$^{13}C_2$]shikimic acid (Fig. 1; Heide et al., 1989), we have shown that the production of 4HB in these cell cultures proceeds exclusively via phenylpropanoid intermediates, and it has been proposed that most benzoic acids as well as ubiquinones are derived from the phenylpropanoid pathway in plants (Pennock and Threlfall, 1983). Furthermore, the conversion of the phenylpropanoid precursors to 4HB was recently characterized (Löscher and Heide, 1994), showing that the reaction sequence from chorismate to 4HB in plants involves up to 10 successive enzymatic reaction steps in cell cultures of *Lithospermum erythrorhizon*.

Escherichia coli, on the other hand, possesses a simpler biosynthetic route to 4HB, which involves the direct conversion of chorismate to 4HB by CPL (Fig. 1b). The cloning of *ubiC*, the gene encoding CPL, was recently reported by our group (Siebert et al., 1992, 1994) and by Nichols and Green (1992). The protein was overexpressed, purified, characterized, and shown to be a soluble protein of 19 kD. It has a K_m value for chorismate of 6.1 μM, a pH optimum at 7.5, and does not require cofactors.

In this study we have expressed the *ubiC* gene in tobacco, thereby introducing a single-step process for the production of 4HB in plants. Chorismate, the substrate of the *ubiC* gene product, is an intermediate of the shikimate pathway. In plants this pathway is localized in the plastid, and the existence of an additional shikimate pathway in the cytosol is controversial (Hrazdina and Jensen, 1992). Therefore, the *ubiC* gene product was targeted to plant plastids by fusing it to a sequence for the chloroplast transit peptide of the small subunit of Rubisco. Expression of these constructs in

[1] This work was supported by the Alfried Krupp-Förderpreis and the Deutsche Forschungsgemeinschaft (grants to L.H.), the Adenauer-Stiftung (grant to M.S.), and the Dr. Hilmer-Stiftung (grant to S.S.)

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Abbreviations: CPL, chorismate pyruvate-lyase; 4HB, 4-hydroxybenzoic acid; MS, Murashige-Skoog.

tobacco led to the accumulation of 4HB glucosides in the transgenic plants.

MATERIALS AND METHODS

Chemicals and Enzymes

Chorismate was purchased as the barium salt from Sigma, and [1,7-$^{13}C_2$]shikimic acid was synthesized as described by Cho et al. (1992). *Taq* polymerase was obtained from Pharmacia or Amersham, and the T4-DNA-ligase and Klenow enzymes were purchased from Boehringer Mannheim. The Sequenase Kit, version 2.0, was obtained from United States Biochemical, and [^{35}S]dATPαS was purchased from Amersham. PCR primers were synthesized by Michael Weichsil Gartner Biotech (Ebergsberg, Germany).

Bacteria, Plants, Cell Cultures, and Media

Escherichia coli XL1Blue, AN92, and JM109 were described by Bullock et al. (1987), Young et al. (1971), and Yanish-Perron et al. (1985), respectively, and *Agrobacterium tumefaciens* strain LBA4404 was described by Hoekema et al. (1983). Cell cultures of tobacco (*Nicotiana tabacum*) were established by cutting sterile-grown seedlings into 1-cm pieces and culturing them on MS medium (Murashige and Skoog, 1962) with callus-inducing hormones (containing per L: 4.46 g of MS salts [Sigma], 30 g of Suc, 0.05 mg of kinetin, 0.5 mg of 2,4-D, and 10 g of Bacto Agar [Difco, Detroit, MI], pH 5.7). After 4 to 6 weeks the generated callus tissues were transferred into 300-mL conical flasks; each flask contained 75 mL of liquid MS medium (containing per L: 4.46 g of MS salts, 30 g of Suc, 1 mg of NAA, and 0.2 mg of 6-benzylaminopurine, pH 5.7). The cultures were maintained in the dark at 25°C and 80 rpm, with subculturing at 14-d intervals. Germination medium contained per L: 4.6 g of MS salts with minimal organic contents [Sigma], 30 g of Suc, and 8 g of Bacto Agar, pH 5.7.

Plasmids and DNA Manipulation

pROK1 and pUC18 were described by Baulcombe et al. (1986) and Yanish-Perron et al. (1985), respectively; pUbiC and pALMU1 were described by Siebert et al. (1994) and Siebert et al. (1992), respectively; and pTSS1-91(#2)-IBI was described by Sugita et al. (1987). DNA manipulation was performed according to the method of Sambrook et al. (1989). The sequencing reactions were performed using the kit from United States Biochemical according to the manufacturer's instructions.

Construction of Plasmids and Transformation of Plants

A PCR product was obtained from plasmid pALMU1 (Siebert et al., 1992) with the primers ubiC-3F (5′-TTA TGT AAC GGA GAG TTC GGC ATG-3′) and ubiC-R (5′-ATT CTG CGT CAG ACT CCA CTC CAT ATT TTT TTC CTC TTA-3′) and was ligated into the *Sma*I site of pUC18 to give plasmid pUbiC. The correct orientation of the insert was confirmed by restriction analysis. The PCR product obtained from the pTSS1-91(#2)-IBI (Sugita et al., 1987) with the primers RBCS-F (5′-CGGAATTCAGATCTAGACAAT ATG GCT TCC TCT G-3′) and RBCS-0R (5′-TGGGGTAC-CGC GCA GCT AAC TCT TCC AC-3′) was digested by *Eco*RI and *Kpn*I. This insert was ligated into plasmid pUbiC, digested with *Eco*RI and *Kpn*I, resulting in plasmid pTP-UbiC. Subsequently, the plasmid pTP-UbiC was digested by *Bgl*II and *Sal*I. The resulting fragment, containing the TP-*ubiC* fusion gene, was ligated to the *Bam*HI site of pROK1 with the compatible *Bgl*II site. The noncompatible *Sal*I and *Bam*HI ends were filled in with Klenow polymerase and blunt end-ligated, resulting in the construction of plasmid p35S-TP-UbiC. The correct orientation of the insert was confirmed by restriction analysis. Transformation of *A. tumefaciens* strain LBA4404 was carried out as described by Höfgen and Willmitzer (1988), and leaf disc transformation and regeneration of *N. tabacum* was performed according to the method of Baumann et al. (1987), which was modified according to the method described by Horsch et al. (1985).

Genomic DNA Gel Blots, RNA Gel Blots, and Immunoblots

DNA was extracted from tobacco suspension cultures as described by Dellaporta et al. (1983). To detect the presence of the *ubiC* gene, PCR analysis was done according to standard procedures (Sambrook et al., 1989) using the

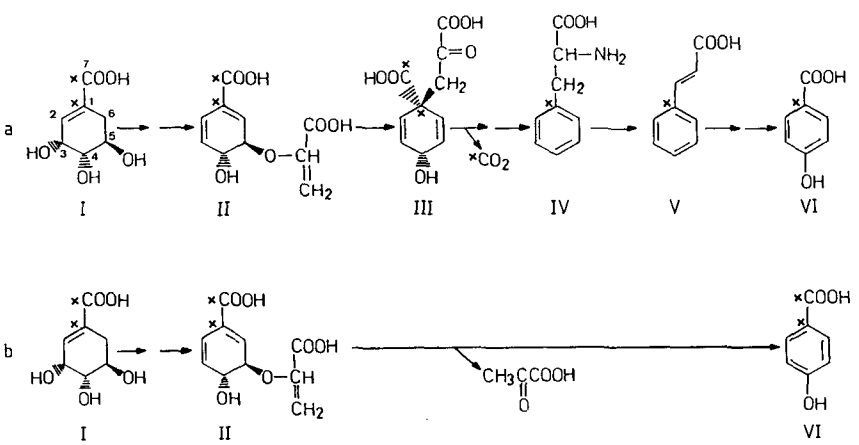


Figure 1. Pathway for biosynthesis of 4-hydroxybenzoate in plants (a) and *E. coli* (b). I, Shikimic acid; II, chorismic acid; III, prephenic acid; IV, phenylalanine; V, cinnamic acid; VI, 4HB. The positions used for ^{13}C labeling (see text) are marked with an x.

primers: 5′-CAC ACC CCG CGT TAA CGC-3′ and 5′-CAT TCT GCG TCA GAC TCC-3′. The calculated size of the amplified band was 531 bp.

Genomic DNA gel blots were prepared by the digestion of tobacco genomic DNA with *Hin*dIII and *Eco*RI, the separation of DNA fragments on an agarose gel, and the transfer to Hybond-N^+ membranes (Amersham). A PCR product was obtained from pUbiC with the primers mentioned above, labeled with [α-^{32}P]dCTP, and used as a probe. Prehybridization and hybridization were performed at 65°C in Rapid-hyb-buffer (Amersham).

RNA was extracted from tobacco suspension cultures as described by Logemann et al. (1987). RNA was separated on a 1.5% agarose gel with 6% formaldehyde following standard procedures (Sambrook et al., 1989). Transfer to Hybond-N^+ membranes and detection were carried out as described below.

Bacterial and plant enzyme extracts were prepared for immunoblots as described above. Plant extracts were applied to a HiTrap blue column (Pharmacia; 10-mL bed volume), and CPL was eluted with a gradient of 0.2 to 2 M NaCl in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM EDTA. Fractions containing the CPL activity, or the corresponding fractions obtained from untransformed control plants, were subjected to SDS-PAGE and blotted onto nitrocellulose membranes according to standard procedures (Sambrook et al., 1989). The UbiC protein was detected using specific antibodies (Siebert et al., 1992) and the BM chemoluminescence western blotting kit (Boehringer Mannheim).

Preparation of Enzyme Extracts and Determination of CPL Activity

Bacterial enzyme extracts were prepared as described by Siebert et al. (1992, 1994). For the extraction of enzymes from plants, tobacco leaves (2 g) were homogenized in an ice-cooled mortar with 2.6 mL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% β-mercaptoethanol, and 200 mg of polyvinylpolypyrrolidone. After the sample was centrifuged (20,000*g*, 10 min) the supernatant was passed over a Sephadex G-25 (Pharmacia) column that was equilibrated with 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 200 mM NaCl; the pH of this buffer was adjusted to pH 8.0 at 37°C. Protein content was determined as described by Bradford (1976), and CPL activity in bacterial extracts was assayed according to the method of Siebert et al. (1992). A modified assay was used for plant extracts, containing in a total volume of 500 μL: 100 nmol of purified chorismate (Siebert et al., 1994), 25 μmol of Tris-HCl, pH 8.0, 5 μmol of EDTA, 100 μmol of NaCl, and 50 μg of protein. Incubations were carried out at 37°C for 15 min, after which reactions were stopped and analyzed by HPLC as described by Siebert et al. (1992). A correction was made for the amount of 4HB formed by the chemical decomposition of chorismate.

Fractionation of Enzyme Extracts from Tobacco Cell Cultures

Cytosolic and plastid fractions were prepared and assayed for the respective marker enzymes, alcohol dehydrogenase, and shikimate dehydrogenase, as described by Sommer et al. (1995).

Isolation and Structural Elucidation of 4HB Glucosides

Fifty grams of fresh leaves was extracted with 120 mL of methanol, and the solution was subsequently concentrated to 16 mL, acidified to pH 2.0 (1 N HCl), and extracted twice with an equal volume of diethyl ether. The ether phase was used for the determination of free 4HB (see below). Aliquots of the aqueous phase were subjected to HPLC on a Multospher 120 RP-18 column (C & S Chromatographie Service, Düren, Germany) using a gradient from 15 to 50% methanol in water:formic acid (99:1, v/v). The two dominant compounds detected at 254 nm were collected and repurified on the same HPLC column using a gradient from 0 to 50% methanol in water:formic acid (99:1, v/v). Evaporation of the solvent produced compounds **1** (10 mg) and **2** (3 mg), which were analyzed by UV spectroscopy and NMR.

The properties of compound **1** [4-*O*-(1-β-D-glucosyl)benzoic acid] were as follows. By UV spectroscopy: λmax: 255 nm (H_2O, pH 6.0); by ^{1}H NMR: (250 MHz) δ_{ppm} (D_2O), 3.1 to 3.8 (m, 6H), 5.09 (d, 1H, J = 7.3 Hz, H-1′), 7.02 (d, 2H, J = 9 Hz, H-3,5), 7.82 (d, 2H, J = 9 Hz, H-2,6); by ^{13}C NMR (100 MHz): δ_{ppm} (CD_3OD): 62.5 (C-6′), 71.3 (C-4′), 74.8 (C-2′), 77.9 (C-3′), 78.3 (C-5′), 101.7 (C-1′), 117.1 (C-3, 5), 125.7 (C-1), 132.7 (C-2, 6), 162.8 (C-4), 169.6 (COO—).

The properties of compound **2** (4HB 1-β-D-glucosyl ester) were as follows. By UV spectroscopy: λmax: 264 nm (H_2O, pH 6.0); by ^{1}H NMR (400 MHz) δ_{ppm} (DMSO-d_6), 3.1 to 3.8 (m, 6H), 5.54 (d, 1H, H-1′), 6.88 (d, 2H, J = 9 Hz, H-3,5), 7.87 (d, 2H, J = 9 Hz, H-2,6); by ^{13}C NMR (100 MHz): δ_{ppm} (DMSO-d_6), 60.6 (C-6′), 69.6 (C-4′), 72.6 (C-2′), 76.5 (C-3′), 77.9 (C-5′), 94.6 (C-1′), 115.4 (C-3,5), 119.8 (C-1), 131.9 (C-2,6), 162.4 (C-4), 164.4 (COO—).

Determination of 4HB Content

To determine the total 4HB content, 1.5 g of fresh leaves was homogenized with liquid nitrogen and extracted at room temperature with methanol (5 mL) using an Ultra-Turrax (Janke and Kunkel, Staufen, Germany) at 8000 rpm for 1.5 h, and the pellet was re-extracted with 2 mL of methanol. The combined extracts were centrifuged at 13,000*g* for 15 min; the supernatant was evaporated and the residue was hydrolyzed with 0.1 N HCl at 80°C for 2 h. 4HB was extracted with ethyl acetate (2 × 1 mL), and the organic phase was evaporated and analyzed for 4HB by HPLC using the same method as for the CPL assays.

To determine the amount of free 4HB, the ether phase obtained during the isolation of the 4HB glucosides (see above) was examined by HPLC, using the same conditions as for the CPL assay. For analysis of the 4HB content in the cell walls, the methanol-insoluble residue was subjected to acid (1 N HCl) or alkaline (1 N NaOH) hydrolysis for 2 h at 95°C and extracted with ethyl acetate, and the organic layer was analyzed by HPLC.

Segregation of the Antibiotic-Resistant Phenotype in the First Self-Fertilized Filial Generation

A total of 468 seeds that were obtained from transformant II was sterilized by 5% $Ca(OCl)_2$, supplemented with 1% Tween 20 (Sigma) for 15 min, and placed on germination medium containing 100 mg/L kanamycin; 95.7% of the seeds germinated. A ratio of 1:3.2 (sensitive:resistant) was observed, and similar results were obtained from the other transformants.

Feeding of [1,7-$^{13}C_2$]Shikimic Acid

A total of 14.08 mg [1,7-$^{13}C_2$]shikimic acid was added to eight flasks, each containing 75 mL of MS medium with transgenic tobacco suspension cultures of transformant II, 4 d after inoculation. After 7 d of incubation (25°C in the dark at 80 rpm) the cells (150 g) were harvested and homogenized in 350 mL of ice-cooled methanol. The methanolic solution was filtered and evaporated to 70 mL, and after the addition of 80 mL of water the solution was extracted with 3 × 100 mL of diethyl ether, and the organic phase was discarded. The aqueous solution was evaporated to dryness, and the residue was taken up in 40 mL of methanol, sonicated, separated from insoluble parts by filtration, and evaporated to dryness. This procedure was repeated; the filtrate was evaporated to dryness after the addition of 4 g of silica gel and applied to a silica gel column (30 g) in ethyl acetate. Elution was carried out with a gradient of methanol (0–20%) in ethyl acetate. Fractions of 20 mL were collected and analyzed for 4-*O*-(1-β-D-glucosyl)benzoic acid by TLC (silica gel F_{254}, acetone:chloroform:formic acid, 5:4:1, v/v), using UV detection. Fractions containing 4-*O*-(1-β-D-glucosyl)benzoic acid were pooled and evaporated to dryness, and the residue was purified by HPLC (RP-18 column; water:methanol:formic acid, 183:15:2, v/v). The fractions containing 4-*O*-(1-β-D-glucosyl)benzoic acid were pooled and evaporated to dryness, and the crystalline residue was recrystallized from 0.2 mL of water to yield 7.6 mg of white crystals.

RESULTS AND DISCUSSION

Construction of Vectors for *ubiC* Expression in Plants

To achieve expression of the bacterial *ubiC* gene in plants, the gene was fused to a plant promoter, to a plant terminator, and to a transit peptide for plastid targeting. Because no suitable restriction site was available upstream of the *ubiC* start codon in the original sequence of *E. coli*, the structural gene was amplified by PCR, and the PCR product was ligated into a pUC18 vector. From this construct, termed pUbiC, the CPL was expressed as a fusion protein with the first 17 amino acids of the *lacZ* gene product (Fig. 2). Subsequently, the gene for the transit peptide of the Rubisco small subunit, rbcs-2A (Pichersky et al., 1986), was amplified by PCR as described in "Materials and Methods." The PCR product was ligated into the pUbiC vector (Fig. 2), and the resulting construct was termed pTP-UbiC. Sequencing of the entire insert confirmed that no mutations had occurred during the PCR amplifications.

To confirm the enzymatic activity of the proteins expressed from these gene fusions, the recombinant plasmids were transformed into the *E. coli* mutant AN92 (Young et al., 1971), which lacks chorismate mutase and therefore allows the accurate measurement of CPL activity (Lawrence et al., 1974). Both constructs caused an increase of CPL activity by 2 or 3 orders of magnitude, as compared with the empty vector, indicating that the expressed proteins were enzymatically active (Table I). However, the construct including the transit peptide gave lower activities than the construct with *ubiC* alone, indicating that fusion with the transit peptide reduces enzymatic activity. However, this finding should not present an obstacle to an expression with high activity in plants, because the transit peptide is expected to be removed after import into the plastid. Using an antibody obtained against UbiC (Siebert et al., 1992) we carried out immunoblots for the fusion proteins produced from pUbiC and pTP-UbiC (Fig. 3A, lanes 3 and 4). The apparent M_r observed for the proteins exceeded the calculated M_r by approximately 10%, which is still in reasonable agreement with the expected value. The lower band observed in lane 4 may be attributed to a degradation product of the original LacZ-TP-UbiC fusion.

ubiC Expression in Transgenic Plants

To achieve transformation into tobacco the gene construct was transferred into the binary vector pROK1 (Baulcombe et al., 1986), which contains a 35S promoter and a *nos* terminator for foreign gene expression, and a kanamycin-resistance gene as a selectable marker (Fig. 2). The binary vector construct was used for *A. tumefaciens* transformation of leaf discs of *N. tabacum* cv Petite Havanna (SR1). Four transgenic plants (I-IV) were regenerated under a selection pressure for antibiotic resistance, with no phenotypic changes observed in comparison with the untransformed control. These plants were examined by PCR (data not shown) and by genomic DNA gel blot analysis (Fig. 3B), and the bacterial *ubiC* gene, which was absent in the untransformed control, was present. Transgenic plant seeds obtained by self-pollination were germinated under sterile conditions in the presence of kanamycin and were examined for segregation of the antibiotic-resistance phenotype. A segregation of approximately 1 (sensitive) to 3 (resistant) was observed (see "Materials and Methods"), as expected for a monogenic dominant trait. Therefore, we concluded that the antibiotic-resistance gene had been stably integrated into the genome at one locus.

RNA gel blots showed, upon hybridization with a *ubiC* probe, a single band of the expected size (approximately 1100 bases), whereas no signal was observed with SR1 control plants (Fig. 3C). The transgenic plants were investigated for CPL activity, and the results are shown in Table I. The transformants had high enzyme activity, exceeding those of a wild-type *E. coli* by a factor of up to 80. As expected, different transformants showed different activi-

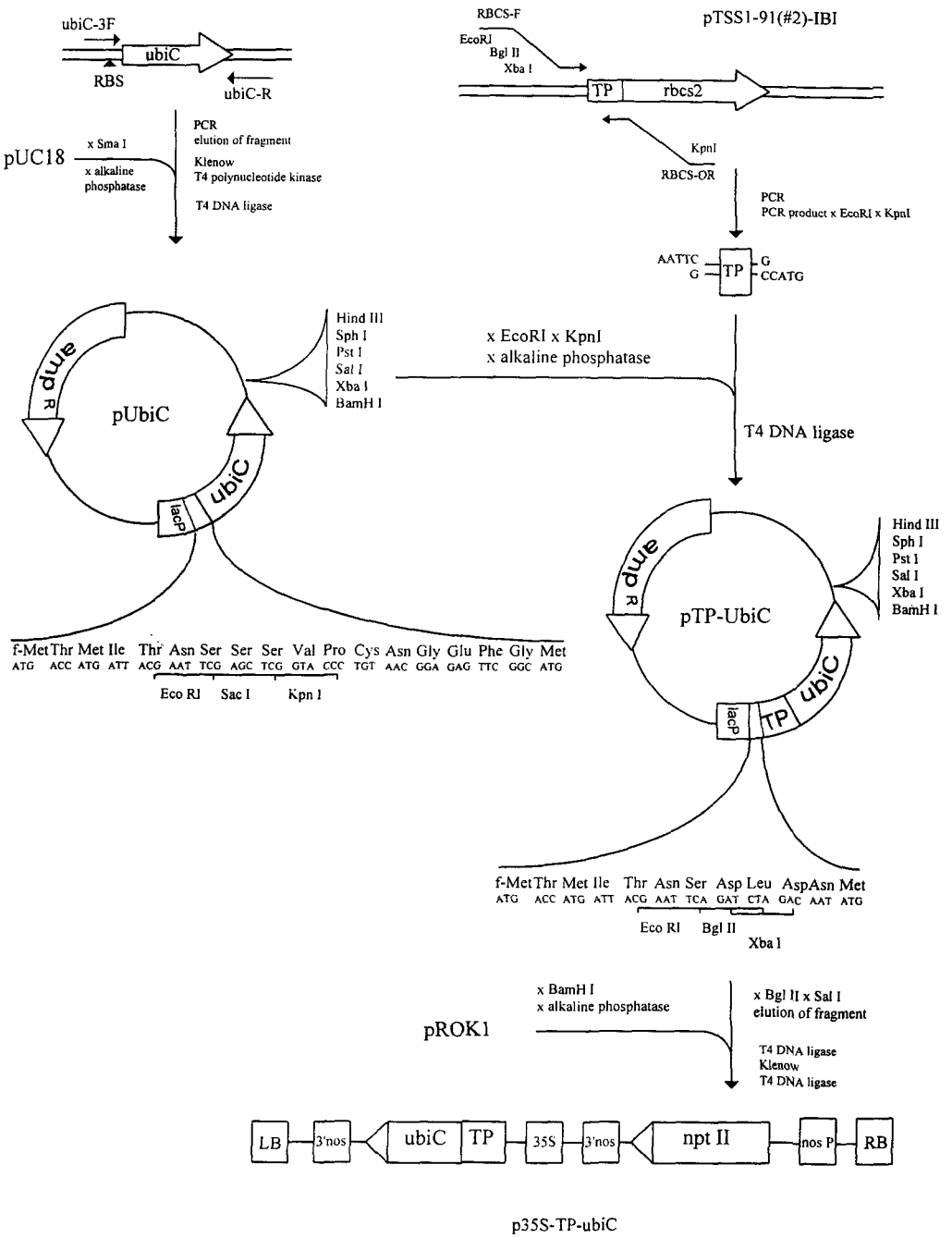


Figure 2. Construction of vectors for the expression of the bacterial *ubiC* gene in tobacco. The nucleotide sequences of the first 18 codons of the *lacZ-ubiC* gene fusion and of the first 12 codons of the *lacZ*-transit peptide-*ubiC* fusion are given. The last codons represent the native start codon of *ubiC* or of the transit peptide, respectively. RBS, Ribosomal binding site; lacP, *lacZ* promoter; amp^R, ampicillin-resistance gene; TP, transit peptide sequence of the small subunit of Rubisco; nptII, neomycin phosphotransferase gene; 35S, cauliflower mosaic virus 35S promoter; nos P, *nos* promoter; 3'nos, *nos* terminator; LB, left border; RB, right border; f-Met, *N*-formyl-methionine. RBSC-F and RBSC-OR, forward and reverse primer (see "Materials and Methods").

ties, presumably because of integration into different positions of the genome.

Immunoblot analysis (Fig. 3A) of the transgenic plants showed a single band at the appropriate position for UbiC, rather than for the TP-UbiC fusion, suggesting that the TP-UbiC fusion protein had been processed by the stromal protease of the plastid. In addition, cell-fractionation experiments with cell-suspension cultures obtained from the transgenic tobacco indicated that CPL activity correlated with the plastid marker enzyme shikimate dehydrogenase

Table I. *CPL activity in E. coli AN92 and in N. tabacum after expression of ubiC gene constructs*

The value obtained with pUC18 is expected to correspond to the activity in a wild-type *E. coli*. Enzyme activity data are mean values ± SD of four independent determinations. 4HB content data are mean values ± SD of at least seven independent determinations.

Transformant	CPL Activity	Content of 4HB Derivatives in Leaves
	pkat/mg protein	*μmol/g dry wt*
E. coli::pUC18[a]	2.7 ± 0.8	–
E. coli::pUbiC(+)[b]	3923 ± 263	–
E. coli::pUbiC(−)[c]	3.8 ± 1.4	–
E. coli::pTP-UbiC	675 ± 66	–
N. tabacum SR1	<1.0	<0.02
N. tabacum transgenic plants		
I	207.8 ± 21.3	17.2 ± 2.0
II	148.4 ± 12.7	15.0 ± 4.7
III	138.6 ± 10.1	14.6 ± 4.6
IV	38.4 ± 8.8	5.9 ± 3.8

[a] Empty vector. [b] Vector with *ubiC* in correct orientation. [c] Vector with ubiC in opposite orientation.

rather than with the cytosolic marker enzyme alcohol dehydrogenase (Table II).

Accumulation of 4-Hydroxybenzoate Derivatives in the Transgenic Plants

Because chorismate, as a metabolite of the shikimic acid pathway, is present in the plastids, the expression of active CPL in this compartment should give rise to the formation of 4HB. Therefore, leaves of the transgenic plants were examined for their 4HB content. Transgenic plants accumulated up to 17.2 μmol 4HB/g dry weight, compared with <0.02 μmol/g dry weight in the untransformed control, and the total content of 4HB detected after acid hydrolysis in the different transgenic plants correlated with the CPL activity of these transformants (Table I).

Without acid hydrolysis very little 4HB (0.2 μmol/g dry weight) was detected in the transgenic plants, showing that the plants accumulate 4HB mainly in the form of derivatives. To identify these 4HB derivatives fresh leaves were extracted with methanol. Two compounds (**1** and **2**) were detected, which were present in the transgenic plants but not in the untransformed control; these compounds were isolated by HPLC. Upon acid hydrolysis (1 N HCl for 60 min at 95°C) both compounds produced 4HB, which was identified by TLC, HPLC, and UV spectroscopy. The presence of the 4HB moiety was also confirmed by ^{1}H NMR and ^{13}C NMR analysis of compounds **1** and **2**, which revealed the signals of a benzoic acid substituted at C-4 (see "Materials and Methods"). In addition, both compounds showed the signals of Glc. The ^{13}C NMR spectra of the two compounds differed from each other in the chemical shifts of the carbon atoms of the carboxyl group and of the C-1 of the benzoate moiety and of the C-1 of Glc (C-1′). From the signal in the ^{1}H NMR spectrum at 5.09 ppm (J = 7.3 Hz; H-1 of Glc) and the signals in the ^{13}C NMR spectrum at 101.7 ppm (C-1′), 125.7 ppm (C-1), and 169.6 ppm (COO), compound **1** was identified as the phenolic glucoside of 4HB, 4-*O*-(1-β-D-glucosyl)benzoic acid (Fig. 4, **1**) (Klick and Herrmann, 1988; Tabata et al., 1988). The corresponding signals for compound **2** were found at 5.54 ppm for H-1 of Glc and at 94.6, 119.8, and 164.4 ppm for the three mentioned carbons. Therefore, compound **2** was identified as the ester glucoside of 4HB, 4HB 1-β-D-glucosyl ester (Fig. 4, **2**) (Klick and Herrmann, 1988). An authentic reference substance for compound **1** was obtained, which gave identical spectroscopic data.

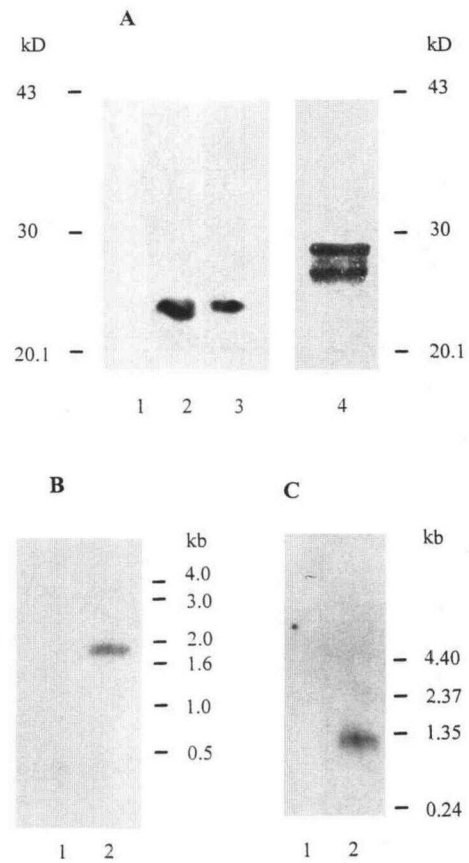


Figure 3. Analysis of tobacco transformed with the *ubiC* gene of *E. coli*. A, Immunoblots; B, genomic DNA gel blot; C, RNA gel blot. Lanes 1, Untransformed control (SR1); lanes 2, transgenic tobacco (transformant II); lanes 3, *E. coli* transformed with pUbiC; lanes 4, *E. coli* transformed with pTP-UbiC. For the genomic DNA gel blots, DNA was digested with *Hin*dIII and *Eco*RI and hybridized with a radioactive probe for the *ubiC* gene. A hybridization band with the expected size of 1.8 kb was detected in the transgenic tobacco (B, lane 2). RNA gel blots were hybridized with the same probe, and a band appeared in the transgenic tobacco at 1.1 kb as expected (C, lane 2). A specific antibody against UbiC was used for the immunoblot (Siebert et al., 1992). The UbiC protein expressed in transgenic plants (lane 2) has a calculated molecular mass of 19.8 kD after removal of the transit peptide; for the intact fusion with the transit peptide, 25.4 kD was calculated. *ubiC* fusion proteins expressed in *E.coli* are shown as a comparison: the LacZ-UbiC fusion (calculated molecular mass 20.5 kD) produced from pUbiC (lane 3); the LacZ-TP-UbiC fusion (calculated molecular mass 26.6 kD) produced from pTP-UbiC (lane 4).

Table II. *Fractionation of a crude enzyme extract from transgenic tobacco plants (transformant II)*

Cell-fractionation and enzyme assays were carried out as described in "Materials and Methods." Data represent mean values ± SD of two incubations.

Fraction	Alcohol Dehydrogenase	Shikimate Dehydrogenase	CPL
	nkat/mg protein	*nkat/mg protein*	*pkat/mg protein*
Crude extract	12.9 ± 0.3	2.65 ± 0.05	35.5 ± 0.5
Cytosol	18.2 ± 0.3	3.45 ± 0.05	32.0 ± 1.0
Organellar extract	3.0 ± 0.1	9.40 ± 0.03	68.0 ± 2.0

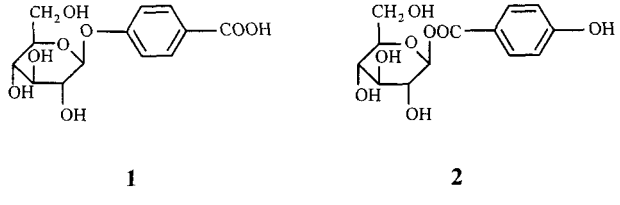
The total amount of 4HB derivatives, calculated as glucosides, was approximately 0.52% of dry weight. The phenolic glucoside **1** represented approximately 69%, and the ester glucoside **2** represented approximately 26% of this total amount of 4HB derivative. Free 4HB accounted for only 1.2% of the total, and another 1.7% was bound to the cell wall and could be released by acid or alkaline hydrolysis (see "Materials and Methods"). The ratio between phenolic and ester glucoside was essentially the same in all four transformants.

The conversion of free 4HB into glucosides may represent a detoxification mechanism of the free phenol, since in previous studies the same conjugates were also produced when various plant species were fed externally 4HB (Cooper-Driver et al., 1972; Klick and Herrmann, 1988).

Incorporation of [1,7-$^{13}C_2$]Shikimic Acid into 4-Hydroxybenzoate in the Transgenic Plants

The 4HB derivatives found in the *ubiC*-transformed plants were most likely formed because of CPL activity. However, the control plants were also expected to produce a certain amount of phenylpropanoid-derived 4HB for the biosynthesis of ubiquinones (Pennock and Threlfall, 1983). Furthermore, some plants and plant cell cultures have been reported to accumulate significant amounts of 4HB derivatives as secondary metabolites (Yazaki et al., 1986; Schnitzler et al., 1992), also derived from the phenylpropanoid pathway (Heide et al., 1989). To quantify to what extent the 4HB in our transgenic plants was a product of the artificially introduced CPL reaction or from the endogenous phenylpropanoid pathway, we carried out feeding experiments with [1,7-$^{13}C_2$]shikimic acid, analogous to published experiments with *E. coli* and *L. erythrorhizon* (Heide et al., 1989; Siebert et al., 1994). Feeding studies were carried out with cell-suspension cultures, which were established from the transgenic tobacco (transformant II). These cell cultures were shown to accumulate the same 4HB derivatives as in the intact transgenic plant (data not shown). After the feeding of the ^{13}C-labeled precursor the phenolic glucoside of 4HB was isolated, crystallized, subjected to ^{13}C NMR analysis (Fig. 5), and compared with the spectrum of an authentic reference substance. If two ^{13}C atoms in adjacent positions were incorporated from our [1,7-$^{13}C_2$]shikimate precursor (Fig. 1), each of the ^{13}C atoms would give a doublet rather than a singlet signal, because of the effect of ^{13}C-^{13}C coupling. The ^{13}C NMR spectrum in Figure 5 shows these doublets, in addition to the original peaks of the carboxyl group (peak a; 169.6 parts per million) and the neighboring ring position (peak b; 125.7 parts per million). This proves that both labeled carbons were incorporated (1.9% enrichment) into 4HB in the transgenic plants and that 4HB was formed as as a result of the introduced CPL reaction rather than by the endogenous phenylpropanoid pathway (Fig. 1). After calculating the integrals of the peaks and the accuracy of the measurement, we concluded that at least 95% of the 4HB, which was found as phenolic glucoside in the transgenic plants, was produced by the CPL reaction that had been introduced by genetic engineering.

The conversion of chorismate to 4HB presents a certain diversion from the natural flow of the shikimate path-



1 **2**

Figure 4. 4-Hydroxybenzoate derivatives accumulated in tobacco plants expressing the bacterial *ubiC* gene. **1**, 4-*O*-(1-β-D-glucosyl)-benzoic acid; **2**, 4HB 1-β-D-glucosyl ester.

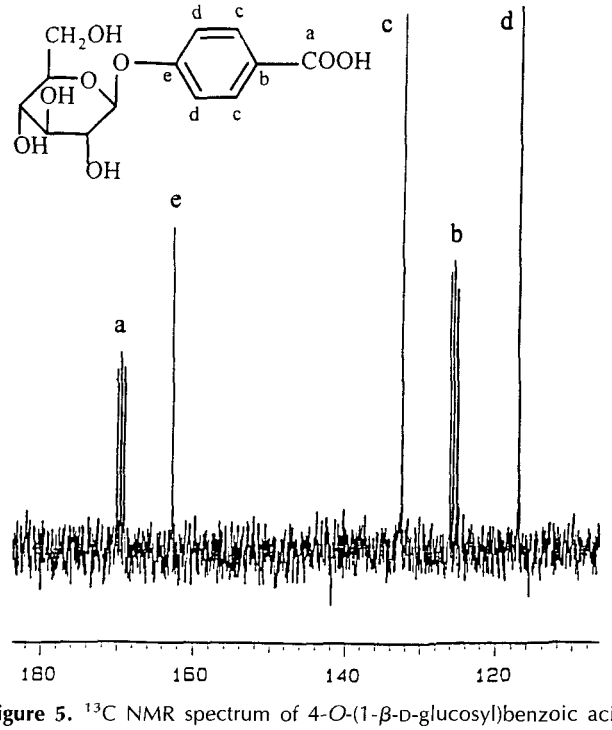


Figure 5. ^{13}C NMR spectrum of 4-*O*-(1-β-D-glucosyl)benzoic acid isolated from transgenic tobacco cell cultures after feeding of [1,7-$^{13}C_2$]shikimic acid. δ_{ppm} (CD_3OD, ^{13}C-depleted; 75 MHz); inverse-gated decoupling. Only the signals of the benzoic acid moiety are shown. Signals of Glc carbons appear between 62 and 102 parts per million. Peak a, 169.6 parts per million; peak b, 125.7 ppm; peak c, 132.7; peak d, 117.1; peak e, 162.8.

way, now producing a secondary metabolite instead of aromatic amino acids. However, the transgenic plants did not show visible phenotypic changes, suggesting that this pathway can be accommodated without seriously affecting other metabolic processes involving shikimate pathway intermediates.

In general, phenolics and especially 4HB esters show antimicrobial activity. In carrot cell cultures, as well as in alfalfa plants, the formation of 4HB can be elicited by treatment with fungal elicitors (Schnitzler et al., 1992; Cvrikova et al., 1993), suggesting a possible role of 4HB derivatives as phytoalexins. In addition, it has been reported that 4HB stimulates the production of pathogen-related proteins in *N. tabacum*, although to a considerably lower extent than salicylic acid (Abad et al., 1988). It will be interesting to test whether the expression of *ubiC* influences the pathogen resistance of plants.

ACKNOWLEDGMENTS

We are grateful to Dr. Meier and Dr. Gruissem (University of California, Berkeley) for the plasmid pTSS1-91-(#2)-IBI, containing the Rubisco small subunit transit peptide sequence; to Dr. C. Gatz (Universität Bielefeld, Germany) for the pBIN-HYG-TX vector; to Dr. A.W. Frahm (Universtiät Freiburg, Germany) for NMR spectroscopy; to Dr. S. Tanaka, Kyoto University (Japan) for samples of 4HB glucosides; to E. Thoma, Freiburg for technical assistance; and to Dr. E. O'Brien and Dr. M. Lange for checking the manuscript.

Received May 13, 1996; accepted July 12, 1996.

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